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Anti-inflammatory Effects of Recombinant Arginine Deiminase Originating from *Lactococcus lactis* ssp. *lactis* ATCC 7962

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Abstract Arginine deiminase (ADI, E.C. 3.5.3.6), one of the arginine deprivation enzymes, exhibits anticarcinogenic activities. The present study investigated the anti-inflammatory activities of the purified recombinant ADI originating from *Lactococcus lactis* ssp. *lactis* ATCC7962 (LADI). LADI dose-dependently inhibited lipopolysaccharide (LPS)-induced upregulation of inducible nitric oxide synthase and the production of nitric oxide in RAW 264.7 murine macrophages. The induction of cyclooxygenase-2 expression and subsequent production of prostaglandin E_2 by LPS was also attenuated by LADI treatment. Moreover, LADI inhibited the production of interleukin-6 in LPS-stimulated RAW 264.7 macrophages. These results indicate that LADI exerts anti-inflammatory effects, which may in part explain its chemopreventive potential.

Keywords: Arginine deiminase, lactic acid bacteria, inducible nitric oxide synthase, cyclooxygenase-2, interleukin-6

Inflammation is a physiological response to tissue damage resulting from chemical irritation, microbial pathogen infection, and wounding [22]. Recent data have also indicated that inflammation is associated with cancer [5], with the key molecular players including inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and interleukin (IL)-6 [1]. iNOS is one of three key enzymes (endothelial NOS, neutral NOS, and iNOS) generating nitric oxide (NO), which is a small-molecule, membrane-permeable gas that mediates many physiological events. iNOS is responsible for the overproduction of NO and is often observed during inflammation and tumor development [18]. COX catalyzes the rate-limiting step in prostaglandin biosynthesis. There are two isoforms of COX: COX-1 produces physiological levels of prostanoids and is constitutively expressed for normal physiological functions, whereas COX-2, the inducible isoform of the enzyme, is induced in many cells during the stimulation of inflammatory mediators such as lipopolysaccharide (LPS) and cytokines [4]. Abnormal expression of COX-2 has been implicated in the pathogenesis of various human malignancies by survival advantage leading to resistance to apoptosis and increased invasiveness or angiogenesis [34]. IL-6 is a typical pleiotropic cytokine that plays an important role in supporting hematopoiesis, regulation of immune responses, generation of acute-phase reactions, and carcinogenesis [10].

Arginine significantly influences the inflammation process and carcinogenesis because it is involved in polyamine synthesis, creatine production, and NO generation. Arginine deiminase (ADI, E.C. 3.5.3.6) is one of most investigated arginine-depletion enzymes. ADI is a key enzyme of the ADI pathway that utilizes arginine as a major nonglycolytic energy source [39]. Previous studies found that ADI originating from *Mycoplasma arginini* (MADI) inhibited cell proliferation and induced apoptosis in arginine auxotrophs such as in hepatocellular, melanoma, leukemia, and prostate cancer cell lines [6, 7, 12, 25]. Phase I and II clinical studies have been performed on hepatocellular carcinoma [11] and melanoma [2]. MADI also exerts anti-inflammatory effects by inhibiting NO synthesis [25] and neutralizing endotoxin [31].

Lactococcus lactis is a lactic acid bacteria (LAB) that is widely used in the manufacture of many fermented dairy products, including cheese [3]. Recently, we found that antiproliferative and ADI activities are positively correlated in six types of LAB tested on the SNU-1 human stomach cancer cell line, and we also found that these effects of LAB were due to the antiproliferative effects of ADI [16]. However, there is no report on the anti-inflammatory effects of ADI originating from *L. lactis* ssp. *lactis* ATCC7962 (LADI). In the present study, we investigated the effects of

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LADI on the expression of iNOS and COX-2 and the production of NO, prostaglandin E_2 (PGE_2), and IL-6 in the LPS-induced RAW 264.7 macrophage cell line.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's minimum essential medium, fetal bovine serum, penicillin, and streptomycin were purchased from Gibco-Invitrogen (Grand Island, NE, U.S.A.). 3-(4,5-Dimethyl-2-thiazol-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from USB (Cleveland, OH, U.S.A.). iNOS and COX-2 antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The enzyme immunoassay (EIA) kit for PGE_2 was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). The enzyme-linked immunosorbent assay (ELISA) kit for IL-6 was purchased from Pharmingen (San Diego, CA, U.S.A.). All other chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

Preparation of LADI

LADI was expressed and purified to homogeneity, and its enzyme activity was determined in our laboratory [13]. Briefly, the *arcA* gene of *L. lactis* ssp. *lactis* ATCC 7962 (NCBI Accession Number DQ364637) that coded LADI was cloned. We constructed pLADI, in which the *arcA* gene was located downstream of the T7 promoter. LADI was expressed by IPTG (1 mM) in *E. coli* BL21 (DE3) harboring pLADI. Anion-exchange and size-exclusion chromatographies were used for the purification. For cell treatment, LADI was dissolved in PBS after dialyzing and freeze-drying (Fig. 1). One unit of the ADI activity was defined as the amount of the enzyme that converted 1 μmol of L-arginine into 1 μmol of L-citrulline every minute at pH 7.2 and 37°C.

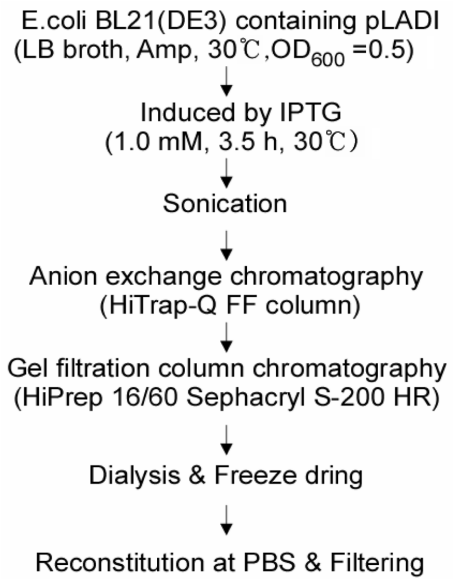

Fig. 1. Flow chart of the procedure for purifying ADI.

Cell Culture

The RAW 264.7 murine macrophage cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Determination of Cell Viability

The MTT assay sensitively measures the normal metabolic status of cells, particularly that of mitochondria, which reflects early cellular redox changes. Macrophages were seeded (5×10^4) in 96 wells and incubated for 24 h. After incubation, the cells were pretreated with LADI at the indicated concentrations for 2 h before incubation with LPS (2 μg/ml) for 24 h. The cells were then treated with the MTT solution (final concentration, 1 mg/ml) for 2 h. The dark-blue formazan crystals formed in intact cells were dissolved in DMSO, and the absorbance at 570 nm was measured with a microplate reader, which reflected MTT reduction. The results are expressed here as the percentage MTT reduction relative to that in control cells.

Determination of Nitrite Production

Macrophages were seeded (5×10^4) in 96 wells and incubated for 24 h. After incubation, the cells were pretreated with LADI at the indicated concentrations for 2 h before incubation with LPS (2 μg/ml) for 24 h, and then the culture medium was harvested. NO production of macrophages was determined by measuring nitrite (which is the metabolite of NO oxidation) as described previously [9]. Briefly, 100 μl of cell culture medium (without phenol red) was mixed with an equal volume of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylene-diamine-HCl], incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured with a microplate reader. $NaNO_2$ was used as the standard.

Determination of PGE_2 and IL-6 Production

Macrophages were seeded (5×10^4) in 96 wells and incubated for 24 h. After incubation, the cells were pretreated with LADI at the indicated concentrations for 2 h before incubation with LPS (2 μg/ml) for 24 h, and then the culture medium was harvested. PGE_2 and IL-6 were quantified according to the manufacturer's instructions.

Western Blotting Analysis

The cells (2×10^5) were first cultured in a 3-cm-diameter dish for 48 h, and then starved in serum-free medium for

another 12 h. The cells were then treated with LADI (80 and 160 mU) for 2 h before they were exposed to 2 μl of LPS for different periods. After treatment, cells were collected by scraping and centrifugation (1,000 ×*g* for 5 min), and the obtained cell pellets were washed with and resuspended in PBS. After centrifugation, cell lysis was performed at 4°C for 30 min in cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF]. The cell lysates were centrifuged at 23,000 ×*g* for 15 min, and the resulting supernatant was stored at −70°C prior to Western blot analysis. The protein concentration in each sample was measured by subjecting lysate protein (30 μg) to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with the protein electrophoretically transferred to a nitrocellulose membrane (Whatman, Clifton, U.S.A.). Protein bands were visualized by a chemiluminescence detection kit (Amersham, Piscataway, U.S.A.) after hybridization with the HRP-conjugated secondary antibody. The relative amounts of proteins associated with specific antibodies were quantified using Image J software (NIH, Bethesda, U.S.A.).

Statistical Analysis

Where appropriate, data are expressed as mean±SD values, and the Student's *t* test was used for single comparisons. A probability value of $p<0.05$ was used as the criterion for statistical significance.

Results and Discussion

Macrophages play a pivotal role in inflammation. LPS is a component of the Gram-negative bacteria cell wall and a type of endotoxin that stimulates macrophages to secrete proinflammatory chemokines and cytokines such as PGE_2, NO, and IL-6 [35–38]. In this study, we examined the effects of LADI on chemokines and cytokines on LPS-treated macrophages. The nontoxic concentration of LADI was determined by using the MTT assay to measure the cytotoxicity of LADI to RAW 264.7 macrophages. LADI did not significantly affect cell viability at concentrations up to 160 mU (Fig. 2), and hence these concentrations were used in the subsequent experiments.

NOS and its product NO play a key role in the pathophysiology of many human diseases, including cancer, cirrhosis, endotoxic shock, atherosclerosis, arthritis, and diabetes [18]. Normally, iNOS is not present in unstimulated cells. However, it is expressed in several pathophysiological conditions, and produces large amounts of NO in response to inflammatory signals induced by LPS treatment [15, 20, 26]. We found that LPS increased the production of nitrite in RAW 264.7 macrophages, which was dose-dependently

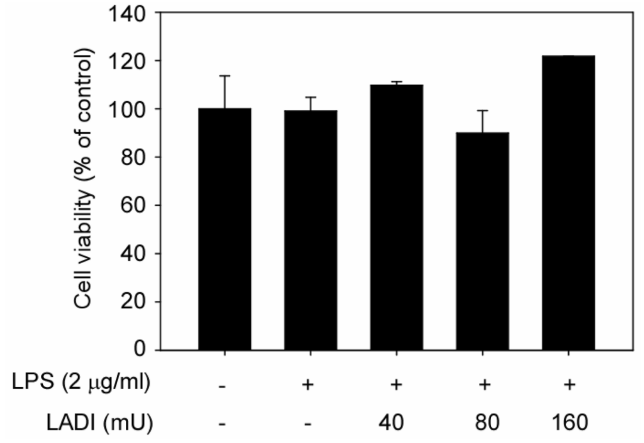

Fig. 2. Cytotoxicity of LADI on RAW 264.7 macrophages.
The cell viability was measured using the MTT assay as described in Materials and Methods. The optical density of formazan formed in control (untreated) cells was taken as 100%. The data are mean and SE values from triplicate tests.

attenuated by treatment with LADI (Fig. 3). Western blotting data revealed that LPS induced upregulation of iNOS expression (Fig. 4), and this was dramatically inhibited by LADI (Fig. 4). The K_m value of iNOS (16±1 μM) is much lower than the intracellular arginine concentrations (range, 0.5–2.0 mM), and hence iNOS is saturated with intracellular L-arginine. However, NO production depends on the exogenous L-arginine concentration [8, 25]. This phenomenon is called the arginine paradox [17], which has been attributed to the intracellular arginine concentration controlling iNOS protein expression [19]. Arginine deprivation can reduce iNOS expression at both the pretranslational and translational levels, depending on the cell type [23].

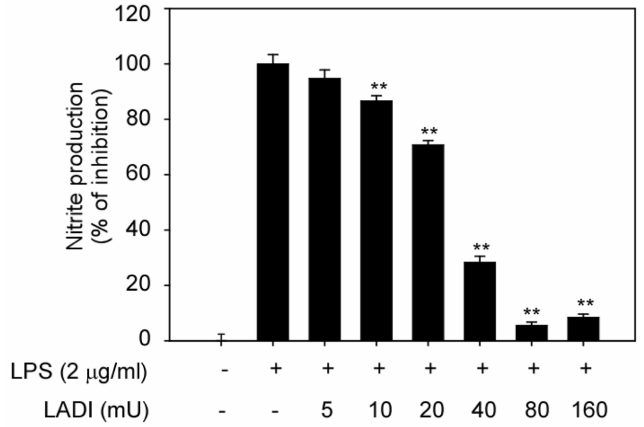

Fig. 3. Inhibitory effect of LADI on LPS-induced generation of nitrite in RAW 264.7 macrophages.
The amount of nitrite was measured using the Griess assay as described in Materials and Methods. The data are mean and SE values from triplicate tests. *$P<0.05$ and **$P<0.01$ indicate statistically significant differences relative to the LPS-treated group.

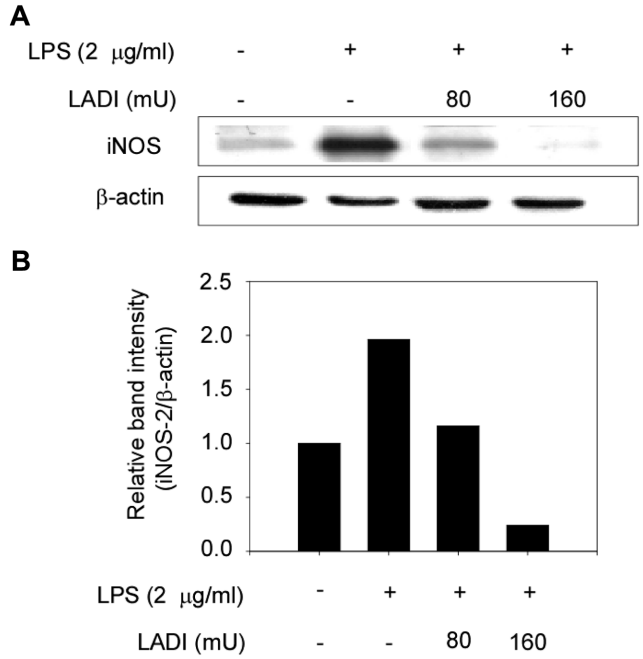

Fig. 4. Inhibitory effect of LADI on LPS-induced upregulation of iNOS expression in RAW 264.7 macrophages.
A. The cells were pretreated with LADI at the indicated concentrations for 2 h before incubation with LPS (2 μg/ml) for 18 h. Equal amounts of total proteins (30 μg) were subjected to 10% SDS-PAGE. The expression of iNOS and β-actin protein was detected by Western blot using specific antibodies. **B**. iNOS and COX-2 protein expression was quantified using an image analyzer.

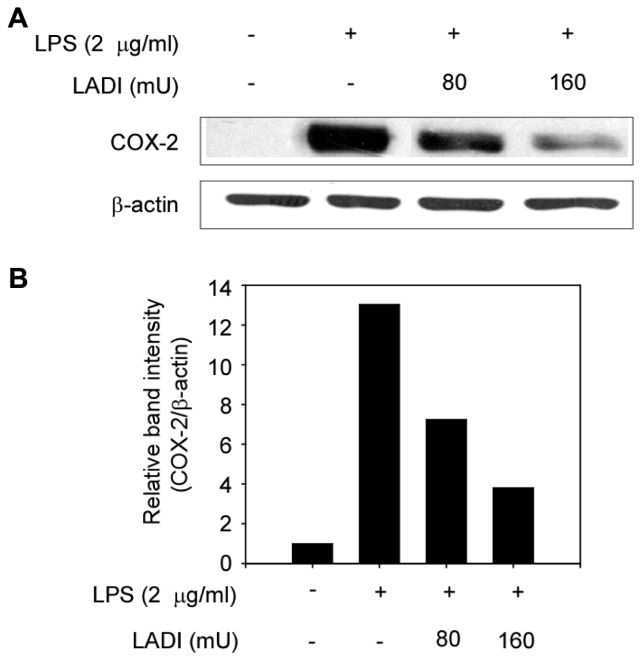

Fig. 6. Inhibitory effect of LADI on LPS-induced upregulation of COX-2 in RAW 264.7 macrophages.
A. The cells were pretreated with LADI at the indicated concentrations for 2 h before incubation with LPS (2 μg/ml) for 12 h. Equal amounts of total proteins (30 μg) were subjected to 10% SDS-PAGE. The expression of COX-2 and β-actin protein was detected by Western blot analysis using specific antibodies. **B**. COX-2 protein expression was quantified using an image analyzer.

Arginine deprivation by ADI may reduce NO generation by reducing iNOS protein expression. MADI has been shown to inhibit the production of nitrite in both macrophages and endothelial cells [25, 30]. Our results clearly demonstrated that LADI inhibits the synthesis of iNOS and the subsequent secretion of NO in LPS-stimulated RAW 264.7 macrophage cells.

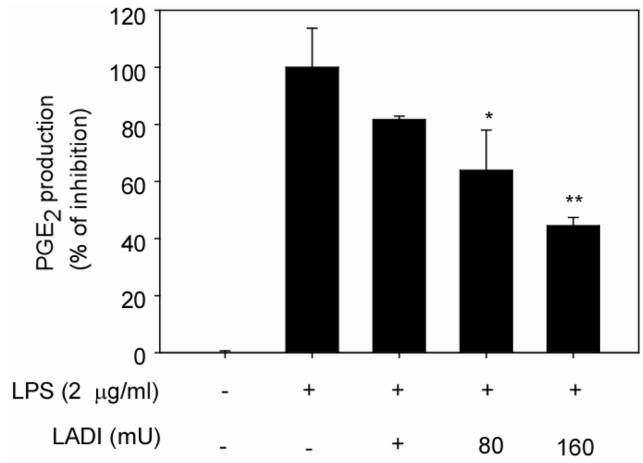

Fig. 5. Inhibitory effect of LADI on LPS-induced generation of PGE_2 in RAW 264.7 macrophages.
The cells were pretreated with LADI at the indicated concentrations for 2 h before incubation with LPS (2 μg/ml) for 24 h. The amount of PGE_2 was measured using a PGE_2 EIA kit in a medium of RAW 264.7 cells. The data are mean and SE values from triplicate tests. $*P<0.05$ and $**P<0.01$ indicate statistically significant differences relative to the LPS-treated group.

PGE_2 promotes cellular proliferation, inhibition of apoptosis, promotion of angiogenesis, stimulation of invasion/motility, and suppression of immune responses [33]. COX-2, which is an enzyme involved in the production of PGE_2, is the key mediator of inflammation [32]. Tumorigenesis is enhanced by overexpression of COX-2 in genetically engineered mice, whereas knocking out COX-2 results in reduced tumor formation and progression [21]. Thus, COX-2 is recognized as a useful molecular target for chemopreventive agents. Similar to iNOS, COX-2 is barely detected in unstimulating cells and is elevated in cells treated with LPS. Treating RAW 264.7 macrophages with LPS for 24 h resulted in the generation of PGE_2 (Fig. 5). LADI at 40, 80, and 160 mU dose-dependently attenuated the production of PGE_2 in LPS-stimulated RAW 264.7 macrophages (Fig. 5). Furthermore, treating RAW 264.7 macrophages with LPS induced upregulation of COX-2 expression, which was inhibited by LADI treatment at 80 and 160 mU (Fig. 6). Increased PGE_2 formation by the induction of COX-2 contributes to the pathophysiology of local and chronic inflammations [34].

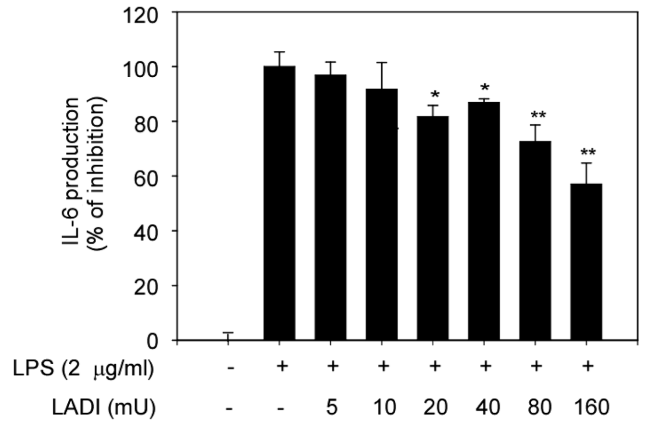

Fig. 7. Inhibitory effect of LADI on LPS-induced production of IL-6 in RAW 264.7 macrophages.
The cells were pretreated with LADI at the indicated concentrations for 2 h before incubation with LPS (2 μg/ml) for 24 h. IL-6 was measured using an IL-6 ELISA kit in a medium of RAW 264.7 cells. The data are mean and SE values from triplicate tests. *P<0.05 and **P<0.01 indicate statistically significant differences relative to the LPS-treated group.

Inhibition of NO by an iNOS inhibitor (N^G-monomethyl-L-arginine) simultaneously inhibits prostaglandin release [29]. Thus, NO inhibition by LADI may contribute to the inhibition of COX-2 expression and PGE_2 generation.

Inflammatory cytokines also play a major role in regulating inflammation and tumor progression. IL-6 is a pivotal proinflammatory cytokine that is regarded as an endogenous mediator of LPS-induced fever. Dysregulation of IL-6 production is implicated in the pathophysiology of several disease processes such as encephalomyelitis, diabetes, bowel disease, and cancer [24]. We found that treating RAW 264.7 macrophages with LPS induced the production of IL-6 (Fig. 7). Adding LADI at 20, 40, 80, and 160 mU simultaneously with LPS for 24 h significantly inhibited the production of IL-6 in RAW 264.7 macrophages (Fig. 7). Inhibition of NO by LADI may affect IL-6 expression owing to the contribution of NO to the generation of IL-6 [27].

LAB are proposed to exert several beneficial health effects, including anticancer activity [28]. However, the effects of the cytoplasmic fraction of LAB on cancer cell proliferation have not been investigated in detail. Recently, we quantified the antitumor capacities of various cellular components (*e.g.*, whole cells, peptidoglycans, and cytoplasmic fractions) from 10 types of LAB by measuring the inhibition of the proliferation of diverse human cancer cell lines. We found that the cytoplasmic fraction of *L. lactis* ssp. *lactis* exerts strong antiproliferative effects on the SNUC2A human colon cancer cell line [14]. Specifically, the antiproliferative and ADI activities were strongly correlated in six types of LAB tested on SNU-1 cells, and we also found that these effects of LAB were due to the antiproliferative effects of ADI [16]. Carcinogenesis is a multistage process that may span more than 20 years, during which there are opportunities to suppress, reverse, or delay critical events in its early and premalignant stages. The promotion stage is closely linked to inflammation and inflammatory tissue damage, and hence the possible anti-inflammatory effects of LADI may be attributable to it acting as an antitumor promoter.

In summary, we have shown that LADI strongly inhibits the generation of NO and PGE_2 in LPS-treated RAW 264.7 macrophages *via* inhibition of iNOS and COX-2 protein expression. LADI also inhibited the production of IL-6 in LPS-treated RAW 264.7 macrophages. Future studies should determine both how LADI inhibits these inflammatory markers and the anti-inflammatory effects of LADI *in vivo*.

Acknowledgments

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